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HYPERBARIC OXYGEN AND SUCCINIC DEHYDROGENASE IN TUBULARIAN DEVELOPMENT[1]

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In 1938, Barth reported that regeneration of *Tubularia* required oxygen and that the size of the regenerating hydranth was directly related to the volume of the gas dissolved in the ambient seawater. His studies embraced the range from 0 ml O_2 per liter to 21.6 ml O_2 per liter. The latter represents the equivalent of 1 atmosphere of pressure and produced the highest rates of regeneration. There have been no studies upon the effects of higher concentrations of oxygen produced by hyperbaroxia either upon regeneration in *Tubularia* or on early development of this hydroid, although his data did not show that the maximal effects of oxygen had been reached. Studies in our laboratory show that 2 to 4 atmospheres absolute of oxygen block differentiation but not the cell migration which occurs during regeneration in this species (Miller, DeSha, Heidger and Miller, 1966).

A striking correlation has been found in *Tubularia* between the early differentiation of organs (tentacles, gonophore buds, perisarc-secreting zone, *etc.*) and the localized increase in succinic dehydrogenase activity (Miller, Hegab and Miller, 1964; Miller *et al.*, 1966). Since Stadie and Haugaard (1945) found that hyperbaric oxygen blocks succinic dehydrogenase activity, a study of the effect of hyperbaroxia is of particular interest in this species in which the enzyme is known to be so closely related to developmental processes.

Material and Methods

Colonies of *Tubularia* sp. were collected from the Cape Cod Canal, transported in iced containers and placed in beakers of running, aerated seawater at 16° C. Hydranths of gravid female colonies were removed and placed in large fingerbowls filled to a depth of 1 ml with filtered seawater (Miller, 1959) approximately 50 hydranths per fingerbowl. During the subsequent 2 to 6 hours at room temperature the gonophores shed their young, first the actinulae and later, younger and younger stages until finally uncleaved eggs were released. Harvesting was done frequently and the young were placed in large volumes of fresh filtered seawater and kept at 15–16° C until the beginning of the experiment. After selection of the stage to be used, the embryos were placed in Petri dishes containing approximately 30 ml of filtered seawater in a cold room which was maintained at 15° C ± 1°. The experimental animals were exposed to gas at the desired pres-

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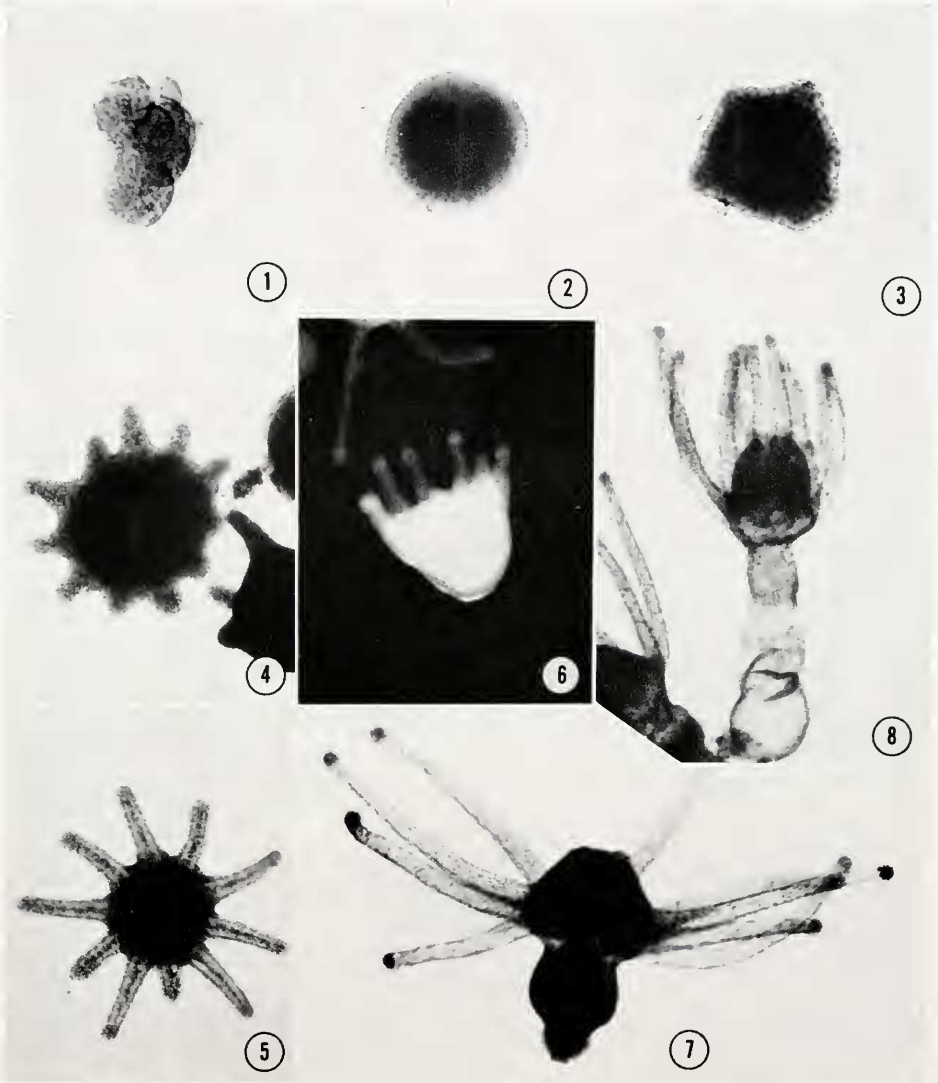

FIGURE 1. Cleavage: this shows the chaotic nature of the cell divisions.

FIGURE 2. Morula: an apparently undifferentiated ball of cells.

FIGURE 3. Polygon: the points on the disk indicate location of future proximal tentacles.

FIGURE 4. Early Star: the tentacles are short.

FIGURE 5. Late Star: the tentacles are elongated but no aboral projection is visible.

FIGURE 6. Basket: growth of the aboral surface has caused the tentacles to project more distally. Dark field photograph.

FIGURE 7. Actinula: the distal tentacles are developing. The aboral surface has differentiated the holdfast (the most distal knob) and the perisarc-secreting growth zone (the subterminal thickened ring).

FIGURE 8. Polyp: the aboral end has attached to the substrate and the growth zone has produced a short stem.

sure in a Bethlehem Co. Table-Top Hyperbaric Chamber. Observations were made and the stages of the embryos were recorded at approximately 24 hour intervals.

At the time of the observations the Petri dishes were refilled with fresh filtered seawater in order to reduce the numbers of bacteria and protozoa. Because deaths in controls increased during the latter part of the season at a time when colonies were heavily infested with protozoa (*cf.* Table IV) the subsequent experiments were performed using pasteurized seawater (20 min at 70° C).

Succinic dehydrogenase activity was assessed by a modification of the method of Nachlas, Tsou, DeSouza, Chang and Seligman (1957) which was used in our previous study (Miller *et al.*, 1964; Miller *et al.*, 1966).

The following stages in the development of *Tubularia* were used in recording observations (Figs. 1–8):

1. Uncleaved egg.

2. Cleavage stages. These are so irregular as to be characterized as chaotic. Cleavage usually begins at the pointed end of the egg and progresses along the external (future aboral) surface more rapidly than along the internal (Fig. 1).

3. Morula, a multicellular ball (Fig. 2).

4. Saucer, a flattened ball wrapped around the gonostyle.

5. Polygon. This is a brief period when the proximal tentacles are first beginning to form. It is named because of its characteristic shape and because the endoderm (but not the ectoderm) of the tentacles shows great activation of succinic dehydrogenase at this stage (Fig. 3).

6. Star. In this stage the proximal tentacles are less than 4 or 5 times as long as wide and there has been an increase in succinic dehydrogenase activity in the ectoderm at the base of the tentacles (Figs. 4 and 5).

7. Basket. Stars transform into baskets as a result of the growth of the aboral surface. As a result the tentacles change from a laterad to distad orientation (Fig. 6).

8. Early Actinula. The growth of the hypostome region and the thickening of the cells which form the perisarc-secreting zone and the adhesive organ give the early actinulae their characteristic appearance.

9. Late Actinula. Late actinulae are distinguished from early stages by their more elongated shape which results both from continued growth of the hypostome region and the perisarc-secreting zone and by the presence of distal tentacles (Fig. 7).

10. Polyp. With the attachment of the actinula to the substrate, the secretion of perisarc and growth of posthydranth tissues the larval period is terminated and its life as a sessile organism commences. Accordingly this has been designated the polyp stage (Fig. 8).

Results

Table I summarizes data on survival of controls and of oxygenated embryos. It shows that exposures to oxygen at hyperbaric pressures between $1\frac{1}{2}$ and 4 atmospheres absolute protected embryos from death during 48 hours of exposure. Although because of their small size, some embryos may have been lost in changing water, care was taken to keep attrition by this route at a minimum and therefore

TABLE I

48 hour survival of control and oxygenated embryos

	Morula	Star and basket	Actinula	Total
Control	63.7%	73.9%	92.9%	73.0%
Oxygen				
1½ AA	80.0%	80.0%	100%	86.7%
2–2½ AA	80.3%	—	—	80.3%
3 AA	77.0%	86.3%	—	83.8%
4 AA	84.0%	95.0%	96.0%	92.5%
Totals:				
Control	63.7%	73.9%	92.9%	73.0%
surv./total	(109/171)	(159/215)	(65/70)	(333/456)
Oxygen	80.7%	89.8%	97.1%	87.5%
surv./total	(138/171)	(193/215)	(68/70)	(399/456)

it can be assumed that very few embryos were lost, and that these losses were approximately equal in control and experimental groups. In addition, fragments of embryos were commonly seen in the control containers but were rare in the oxygenated lots. As a consequence it was concluded that the differences in numbers between the hyperbaric and control animals were real and represented differences in death rates in the two groups. It is to be noted that survivals were lowest and protection by oxygen greatest in the early embryos. When pasteurization was used in addition to filtration survivals were highest in both oxygenated and controls (Tables IV and V). If survival is used as a criterion it may be concluded that hyperbaric oxygen is beneficial within the range tested (1½–4 atmospheres).

TABLE II

Efects of oxygen at 1½ atmospheres absolute on development of tubularian embryos

Before expt.		Treatment	At 48 hours		
			Actinulae		Dead or lost
Stage	No.		Early	Late and polyp	
Morula	20	Control	0	11	9
	20	O_2–1½ AA	0	16	4
Star and Basket	20	Control	0	15	5
	20	O_2–1½ AA	0	16	4
Actinula	20	Control	5	15	0
	20	O_2–1½ AA	0	20	0
Totals	60	Control	5	41	14
	60	Oxygen	0	52	8

TABLE III

Effects of oxygen at 2 and 2½ atmospheres on development of tubularian morulae

Treatment	No. of morulae	At 48 ± hours						
		Morula	Polygon	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Control	28	4	1	0	4	*8*	4	7
O_2–2 AA	28	4	1	*9*	4	0	0	10
Control*	38	3	1	0	5	*15*	0	14
O_2–2½ AA*	38	9	3	*16*	5	0	0	5
Totals								
Control	66	7	2	0	9	*23*	4	21
O_2–2+2½	66	13	4	*25*	9	0	0	15

* Two lots.

Table II summarizes the effects of oxygen at 1½ atmospheres on the development of embryos and larvae of three different stages: (1) morula, (2) star and basket and (3) actinula. The data suggest that morulae but not later stages may be affected by 1½ atmospheres of oxygen.

Table III shows that 2 or 2½ atmospheres of oxygen are definitely inhibitory. None of the morulae subjected to 2 atmospheres reached late actinula stage whereas 8 controls reached this stage and 4 attached to the substrate and became polyps. In this and subsequent tables the italics indicate the largest group. With 2½ atmospheres of oxygen, development was slowed greatly and also appeared to be blocked at the early actinula stage, with the majority of the embryos in

TABLE IV

Effects of oxygen at 3 atmospheres absolute on development of tubularian embryos

Before expt.		Treatment	At 48 hours						
Stage	No.		Morula	Polygon & star	Basket	Actinula		Polyp	Dead or lost
						Early	Late		
Morula	35	Control	0	0	0	1	*12*	1	21
	35	O_2–3AA	0	5	*21*	1	0	0	8
Star (2 lots)	95	Control	0	0	0	7	*26*	17	45
	95	O_2–3AA	0	9	*44*	29	0	0	13
Totals	130	Control	0	0	0	8	*39*	18	65
	130	O_2–3AA	0	5	*74*	30	0	0	21

the basket stage at 48 hours. Fifteen (62.5%) of the surviving controls were late actinulae at the time they were recorded.

Oxygen at 3 and 4 atmospheres (Tables IV and V) inhibits the later differentiation of the embryos with the majority (67.8%) arrested at the basket stage and most of the remainder at the early actinula stage. There were two late actinulae and no polyps. Indeed, it appeared that the larvae were unable to attach to the substrate because of deficiency in adhesive organ development.

TABLE V

Effects of oxygen at 4 atmospheres absolute on tubularian embryos in pasteurized seawater*

Before expt.		Treatment	At 48 hours						
Stage	No.		Morula	Star	Basket	Actinula		Polyp	Dead or lost
						Early	Late		
Morula	50	Control	0	1	14	*24*	0	0	11
	50	O_2–4AA	*21*	15	6	0	0	0	8
Star	50	Control	—	0	5	*19*	8	5	13
	50	O_2–4AA	—	10	*36*	2	0	0	2
Basket	50	Control	—	—	0	17	*20*	10	3
	50	O_2–4AA	—	—	15	*32*	0	0	3
Actinula	50	Control	—	—	—	2	6	*42*	0
	50	O_2–4AA	—	—	—	*32*	15	1	2
Totals	200	Control	0	1	19	*62*	34	*57*	27
	200	O_2–4AA	*22*	25	*57*	*66*	15	1	14

* 20 minutes at 70° C.

To test whether the inhibition was reversible or whether it reflected merely a moribund condition, 18 of these embryos, 6 each in early and late basket and early actinula stages, were removed from the chamber at 48 hours and were permitted to recover in the cold room. After 24 hours there were 2 late actinulae and 16 polyps. Because of the small numbers groups of 50 embryos were tested. This series (Table VI) demonstrates inhibition by 4 A.A. of oxygen similar to that shown in Table V. In addition, after 24 hours in sea water at normal pressure the oxygenated embryos were still in the early actinula stage although the controls had continued to develop. However, by 36 hours all 48 oxygenated embryos had become attached polyps. Again, the embryos tested in the basket stage showed fewer deaths than did the morulae.

In most of the experiments records were kept throughout an extended period. Table VII is a summary of one of the two typical experiments with 2½ atmospheres and illustrates the fact that there was an appreciable time lag in the inhibition produced by excess oxygen. No evidence of inhibition was seen after 24 hours of treatment. However, by 48 hours blockage was nearly 100% in the hyperbaric

TABLE VI

Reversibility of inhibition of development by removal from hyperbaric oxygen

Stage before expt.	Treatment	At 48 hours						
		Morula	Star	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Morula	Control	0	0	0	0	2	0	*48*
	0_2–4AA	*28*	10	8	1	0	0	3
Basket	Control	0	0	0	0	*31*	12	7
	0_2–4AA	0	0	0	*48*	0	0	2
		24 Hours after Removal						
Morula	Control	0	0	0	0	0	0	*50*
	Air	*15*	12	9	1	0	0	13
Basket	Control	0	0	0	0	1	*37*	12
	Air	0	0	0	*48*	0	0	2
		36 Hours after Removal						
Morula	Control	All dead						
	Air	Unchanged—beginning to disintegrate						
Basket	Control	0	0	0	0	0	*20*	30
	Air	0	0	0	0	0	*48*	2

embryos and larvae. By contrast, the controls continued development. Twelve out of fifteen surviving controls had transformed into polyps by 76 hours and all but one had transformed by 116 hours.

Embryos and larvae from both control dishes in air and experimental in hyperbaric oxygen were tested for succinic dehydrogenase activity using the modification of the method of Nachlas *et al.* (1957) mentioned in Material and Methods. Estimations of activity are summarized in Table VIII. The controls exhibited a progressive increase from polygon to late actinula in overall staining with the blue formazan, as had been observed earlier (Miller *et al.,* 1964), with localized centers of activity shown by each group of organs when they began to differentiate. The most prominent of these were used for rating the concentration of the formazan on a scale of − to ++++.

Below is a list, in order of their appearance, of the regions of increased succinic dehydrogenase activity: (1) the endoderm at the tips of the developing proximal tentacles (from the end of the morula stage through early actinula), (2) the ectoderm around the bases of the proximal tentacles (from star to late actinula), (3) the ectoderm of the perisarc-secreting zone (from late basket through polyp), (4) the ectoderm of the future gonophore region (from early actinula through

TABLE VII

Development of inhibition by hyperbaric oxygen ($2\frac{1}{2}$ AA)

Treatment	Time (hours)	Morula	Polygon + star	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Control	24	7	6	7	0	0	0	0
Oxygenated		4	9	7	0	0	0	0
Control	48	2	1	0	2	*11*	0	4
Oxygenated		4	3	*10*	3	0	0	0
Control	76	0	0	0	2	1	*12*	5
Oxygenated		4	2	*10*	3	0	0	1
Control	116	0	0	0	1	0	*13*	6
Oxygenated		3	4	*8*	2	0	0	3

20 Morulae in each lot, stages recorded at 24 hour intervals.

polyp), and (5) the ectoderm of the holdfast (from early actinula through late actinula).

By contrast, the embryos which were subjected to hyperbaric oxygen showed less overall development of the blue formazan, and were particularly deficient in the

TABLE VIII

Effects of hyperbaric oxygen at 3 atmospheres absolute upon succinoxidase activity

Stages		Endoderm of tentacles	Ect. at base of tentacles	Ect. of gonophore region	Ect. of peri-arc zone	Ect. of holdfast zone
Polygon	Control	++	−	−	−	−
	Oxygenated	+	−	−	−	−
Basket	Control	+	++	−	+	−
	Oxygenated	−	−	−	−	−
Early Actinula	Control	+	++	++	+++	++
	Oxygenated	−	−	−	−	−
Late Actinula	Control	−	++	+++	++++	++
	Oxygenated	−	−	−	+?or−	−
Attached (hydranth + stem)	Control	−	++	+++	++	−
	Oxygenated*	0	0	0	0	0

* Did not reach this stage.

TABLE IX

Effects of increased pressure (nitrogen added—oxygen unchanged)

Before expt.		Treatment	At 48 hours						
Stage	No.		Morula	Star	Basket	Actinula		Polyp	Dead or lost
						Early	Late		
2 A.A. of 10% O_2 + 90% N_2									
Morula	75	Control	0	0	1	10	14	*36*	14
	75	2 A.A.	0	0	0	14	8	*27*	26
Basket	25	Control	0	0	0	1	0	*22*	2
	25	2 A.A.	0	0	0	1	1	*23*	0
1 Atmosphere Air + 3 Atmospheres N_2									
Morula	50	Control	15	6	8	0	0	0	*21*
	50	4 A.A.	10	12	0	0	0	0	*28*
Basket	50	Control	0	0	0	0	8	*34*	8
	50	4 A.A.	0	0	0	0	13	*28*	9

loci of intense staining which accompanied the differentiation of intense staining which accompanied the differentiation of the controls. The few larvae that reached late actinula stage showed slight evidence of activity of succinic dehydrogenase in the perisarc-secreting zone (rated +) but in all cases this was far less than that in controls in which the staining was the most intense of any region at any stage of development (rated ++++, Table VIII).

Two experiments were performed to determine whether pressure itself influenced these results. In the first the experimental embryos were exposed to 2 atmospheres of 10% O_2 + 90% N_2 and in the second, to 1 atmosphere of air plus 3 atmospheres of nitrogen. In Table IX it is seen that with normal oxygen and pressure increased with nitrogen there was no difference between experimental and control development. Also, there was no evidence of nitrogen narcosis in this material.

DISCUSSION

These experiments indicate that Barth's highest oxygen levels were very near those which are inhibitory and that at pressures between 1 and 2 atmospheres the toxic effects of oxygen begin to overbalance the stimulatory effects.

Oxygen toxicity has been recognized ever since the classic studies of Paul Bert (1878), and the basic concepts regarding its action are little changed today from those he proposed over 90 years ago. From his extensive studies on bacteria, plants and lower animals as well as mammals, he concluded that elevated pressures

of oxygen were toxic because they inhibited metabolic activities of cells. A detailed review of work in this field was published in 1968 by Haugaard.

The inactivation of succinic dehydrogenase found in our histochemical studies confirms for a hydroid the biochemical findings on mammals (Lehman, 1935; Libbrecht and Massart, 1937; and Stadie and Haugaard, 1945). Although Libbrecht and Massart (1937) found inactivation of succinic dehydrogenase, they attributed this to be secondary to the effects on cytochrome oxidase and the formation of "oxygen actif" which has not been confirmed by later studies. Stadie and Haugaard (1945), who made a thorough study of the situation, found that the mechanism of inactivation is that of oxidation of the active sulfhydryl groups to the inactive thiol form. They reported that the inactivation was reversible and that reactivation of the enzyme could be achieved by incubation with cysteine or glutathione. In Table VI is documented that fact that although the blockage of development of *Tubularia* by 4 atmospheres of pressure was unchanged at 24 hours after removal from the chamber the embryos had recovered when observed at 36 hours. It will be of interest to determine if the inhibition of development and the inactivation of succinic dehydrogenase in tubularian embryos and larvae can be prevented by appropriate administration of the reduced SH compounds glutathione and cysteine.

The lag period of 24 hours which was found before the appearance of symptoms is a characteristic of oxygen toxicity as studied in mammals. This together with its reversibility upon return to atmospheric pressures, even at an advanced stage, is consistent with the hypothesis that the toxicity of hyperbaric oxygen is caused by the oxidation of certain essential enzymes or cofactors. It is assumed that under normal conditions these are present in excess and that they can be reactivated or resynthesized rapidly as soon as oxygen tensions fall to normal levels (Haugaard, 1965). It is possible that in these very small embryos the reactivation may be inadequate in some cases for development to continue.

The protection against death which was noted in these experiments requires comment. Hyperbaric oxygen has been shown to inhibit the growth of bacteria and of protozoa (Caldwell, 1965; Elliott, Travis and Bak, 1962; Towers and Hopkinson, 1965). Since a wide variety of these organisms inhabits *Tubularia* colonies and some are known to inhibit regeneration or to be parasitic, it is possible that inhibition of proliferation of these would result in the survival of greater numbers of embryos in the hyperbaric chamber.

On the other hand, the egg of *Tubularia* does not contain a large amount of stored energy in the form of yolk. As a result the possibility also exists that the inhibition of enzyme activity, as indicated by the succinic dehydrogenase estimations, prevented the early exhaustion of energy stores and thereby prevented death from inanition.

Finally, the fact should be emphasized that hyperbaric oxygen, which so completely (but reversibly) blocks differentiation, has no demonstrable effect upon cytochrome oxidase, carbonic anhydrase and many other enzymes (Haugaard, 1965). On the other hand this treatment has been shown to inactivate the free sulfhydryl groups of the SH-enzymes including succinic dehydrogenase.

It was concluded from the present study that hyperbaric oxygen blocks succinic dehydrogenase activity in developing *Tubularia,* and that since the parallelism

between inhibition of differentiation and the reduction in succinic dehydrogenase activity is so precise, that there is a high degree of probability of a cause-effect relationship between the two groups of data.

These results have significance in the analysis of development of this species since they implicate certain groups of enzymatic processes as having key roles in certain aspects of differentiation. By combining biochemical inhibition or stimulation with blockade of enzymatic processes by hyperbaric oxygen a clearer understanding of embryonic differentiation may be anticipated.

Summary

1. Hyperbaric oxygen reduced deaths of tubularian embryos and larvae in standing seawater.

2. Hyperbaric oxygen blocked differentiation at pressures of 2, 2½, 3 and 4 atmospheres absolute. At 1½ atmospheres it had little or no effect.

3. The blockade occurred between 24 and 48 hours after early actinula or late basket stages were introduced into the chamber. The blocked embryos were unable to secrete holdfast material or produce perisarc.

4. After 48 hours at 4 atmospheres the block was usually completely reversible during 24 to 36 hours after removal. Even after 72 hours a few blocked larvae attached and transformed into polyps when removed from the hyperbaric chamber.

5. Histochemical visualization showed an overall reduction of succinic dehydrogenase activity in all embryos subjected to hyperbaric oxygen. Localized zones of high activity, which in controls were associated with the differentiation of tentacles, gonophore buds, perisarc-secreting region and adhesive organ, failed to develop.

6. Since hyperbaric oxygen reversibly blocks certain enzyme systems but has no effect upon others, this method can prove useful in the biochemical analysis of developmental processes.

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